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Cloning and characterization of myogenic regulatory genes in three Ictalurid species

D. J. Gregory, G. C. Waldbieser and B. G. Bosworth

US Department of Agriculture-Agricultural Research Service, Catfish Genetics Research Unit, Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS 38776, USA

Summary

We report sequence, tissue expression and map-position data for *myogenin*, *MYOD1*, *myostatin* and *follistatin* in three Ictalurid catfish species: channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*) and white catfish (*Ameiurus catus*). These genes are involved in muscle growth and development in mammals and may play similar roles in catfish. Amino acid sequences were highly conserved among the three Ictalurid species (>95% identity), moderately conserved among catfish and zebrafish (approximately 80% identity), and less conserved among catfish and humans (approximately 40–60% identity) for all four genes. Gene structure (number of exons and introns and exon–intron boundaries) was conserved between catfish and other species for all genes. *Myogenin* and *MYOD1* expression was limited to skeletal muscle in juvenile channel catfish, similar to expression patterns for these genes in other fish and mammalian species. *Myostatin* was expressed in a variety of tissues in juvenile channel catfish, a pattern common in other fish species but contrasting with data from mammals where *myostatin* is primarily expressed in skeletal muscle. *Follistatin* was expressed in juvenile catfish heart, testes and spleen. All four genes contained polymorphic microsatellite repeats in non-coding regions and linkage analysis based on inheritance of these microsatellite loci was used to place the genes on the channel catfish linkage map. Information provided in this study will be useful in further studies to determine the role these genes play in muscle growth and development in catfish.

Keywords catfish, expression, *follistatin*, mapping, *MYOD1*, *myogenin*, *myostatin*, sequence.

Introduction

Channel catfish (*Ictalurus punctatus*) farming is the largest sector of the US aquaculture industry (USDA 2002). In addition to their economic value, Ictalurid catfishes provide an interesting model for studying muscle growth in fish. Ictalurid species differ substantially for many traits including growth and meat yield (Pflieger 1975; Dunham *et al.* 1993; Reilly & Lochmann 2000; Argue *et al.* 2003), but interspecific hybrids produce fertile gametes. Therefore, use of interspecific hybrids to produce backcross or F_2 populations could facilitate identification of sequence polymorphisms associated with differences in phenotypic performance. In addition, Ictalurid catfish species generally tolerate a wide range of environmental conditions such as temperature, dissolved oxygen and ammonia levels (Tucker & Robinson 1990; Dunham *et al.* 1993) and evaluation of environmental influences on gene expression and subsequent phenotypic changes could provide insight into the physiology of muscle growth in catfish.

Recent studies have identified genes important in muscle growth and development in mammals (Sabourin & Rudnicki 2000; Lee & McPherron 2001; Armand *et al.* 2003). *Myostatin*, a member of the transforming growth factor (TGF)-β family of secreted growth and differentiation factors, is a potent regulator of skeletal muscle growth in mammals (McPherron *et al.* 1997; Lee & McPherron 2001). Although *myostatin* has been sequenced in several fish species (McPherron & Lee 1997; Ostbye *et al.* 2001; Roberts & Goetz 2001; Rodgers & Weber 2001; Kocabas *et al.* 2002) its role in muscle growth in fish is unclear. *Follistatin*, a protein that binds members of the TGF-β superfamily and regulates their activity (Link & Nishi 1997; Amthor *et al.* 2002), can be a *myostatin* antagonist. Transgenic mice overexpressing *follistatin* exhibit greatly increased muscle

Address for correspondence

B. G. Bosworth, US Department of Agriculture-Agricultural Research Service, Catfish Genetics Research Unit, Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS 38776, USA.
E-mail: bbosworth@ars.usda.gov

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mass (Lee & McPherron 2001). *MYOD1* and *myogenin*, members of the myogenic regulatory factor (MRF) family of genes, are essential to the initial formation and differentiation of skeletal muscle (Emerson 1990; Krempler & Brenig 1999; Sabourin & Rudnicki 2000) and are expressed in unique spatial and temporal patterns in developing skeletal muscle (Du *et al.* 2003). Because of their key roles in myogenesis, *myogenin* and *MYOD1* are frequently used as markers of muscle cell proliferation and differentiation. Therefore, the objective of this research was to sequence and characterize *myogenin*, *MYOD1*, *myostatin* and *follistatin* in three Ictalurid catfish species: channel catfish (*I. punctatus*), blue catfish (*I. furcatus*) and white catfish (*Ameiurus catus*). This information will be useful in studying muscle growth and development in catfish.

Materials and methods

Animals

All experimental fish were raised in the hatchery at the National Warmwater Aquaculture Center, US Department of Agriculture, Catfish Genetics Research Unit Facility in Stoneville, MS, USA. All research was performed under the IACUC approved protocol 64-F-0006 in the Catfish Genetics Research Unit.

Cloning and sequencing

Total RNA was isolated from tissue of killed (200 ppm; Finquil, Argent Chemical Laboratories, Redmond, WA, USA) channel, blue and white catfish tissues using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). Total RNA was stored at −80 °C and then 1 µl aliquots were converted to cDNA using commercially available kits (Cloned AMV First Strand cDNA synthesis kit, Invitrogen, Carlsbad, CA, USA; iScript cDNA synthesis kit, Bio-Rad, Hercules, CA, USA). All primers for these experiments were synthesized by ResGen (Invitrogen) or Integrated DNA Technologies (Coralville, IA, USA). Primers (Table 1) for initial amplification and cloning of catfish *myogenin* and *MYOD1* were based on areas of conserved sequence from other fish and mammalian species. These primers were used to amplify fragments from a channel catfish fry cDNA library. Reaction conditions were 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton-X, 1.2 mM $MgCl_2$, 200 µM dNTP, 200 nM each primer, 0.5 U *Taq* DNA Polymerase (in storage buffer B; Promega, Madison, WI, USA) and 1/20 of the cDNA synthesis reaction as template (thermal cycling protocol listed in Table 1). Resulting fragments were cloned into pCR4-TOPO vector (Invitrogen) and sequenced using a Big Dye® Terminator v 3.1 Cycle Sequencing on an ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Gene identify of subclones was confirmed by sequence homology with known

Table 1 Primer sets and amplification conditions for initial fragment amplification, BAC cloning, tissue expression and genotyping.

Gene	Strand	Initial fragment	BAC cloning	Tissue expression	Genotyping
Myogenin	S	CCGGGYCARTGYCTNCCMTGGGC	AGGACGTGAAAGAGCAAGTG	GGTGGAGGTCCTCAGAAGTGCTAT	GTTTCTACTGAGTGGATCTGCTAG
	A	GCCTCRAAGGCYTCRTTNACYTT	GTATAGGCTTTCTGGAACTGG	TCAGCAGATCTTCATGGGTGGAGC	TATGTAACAGTTTTCTGGATG[1]
MYOD1	S	TGCCTGCTGTGGGCATGCAA	GGATAAACTCTGGGCTACATCT	GGAGACGCCTGAGCAAGGTCAAC	TGTCAGTGTTGCCATATGTG[1]
	A	ATCTCCACTTTGGGCAGCCT	CGTTCTCTGAAATGTTGTGGATG	TCTTCCGGACGGTCGAGCA	GTTTATATCTGCAGTGGTTATTCTCTG
Myostatin	S		CTCTCTGAGACCTGACCTGG	GTCATGAGCATGGCCGCCGAGC	GTTTGGCAGAGTTCTAACAGAAATG
	A		CCTTGCTTGCTGCTATTCTGGTTC	ATTCGTACGTGCCTTCTCCCGTCTT	AAAGGTCAGAGTGTATGGTTAG[1]
Follistatin	S		ATGACAGAGCCCCGCTGGGATCAGT	TGTGATAACGTGGACTGTGCTC	AGTGTGTTCTTCTGAGATGAG[1]
	A		CCCCGCATTAAAACACAAACCGCGT	TGTCCCACAGACACTTCTTTCCGT	CTACAGACAGCTCGCTCCCG
18SrRNA	S			GAGAAACGGCTACCACATCC	
	A			GATACGCTCATTCCGATTACAG	
Amplification protocol		94 °C for 2 min; 35 cycles of	95 °C for 5 min; 30 cycles of	94 °C for 2 min; 35 cycles of	95 °C for 15 min; 25 cycles of
		94 °C for 30 s, 55 °C for 1 min,	95 °C for 5 min, 57 °C for 1 min,	94 °C for 30 s, 55 °C for 1 min,	94 °C for 30 s, 62 °C for 30 s,
		72 °C for 1 min; 72 °C for 5 min	72 °C for 2 min; 72 °C for 7 min	72 °C for 1 min; 72 °C for 5 min	68 °C for 1 min; 72 °C for 10 min

S, sense orientation; A, antisense orientation.
[1]The sequence 'GAGTTTTCCCAGTCACGAC' was added to the 5′-end to facilitate fluorescent labelling.

genes. A channel catfish EST clone (BE469120) containing partial sequence for *follistatin* was available in dbEST (Cao *et al.* 2001).

Channel catfish-specific primers were then used to identify positive clones containing the *myogenin*, *MYOD1* and *follistatin* genes from a channel catfish BAC library via polymerase chain reaction (PCR)-screening of pooled colonies (Quiniou *et al.* 2003; Table 1). The BAC clones were directly sequenced by primer walking (Waldbieser *et al.* 2003) to determine genomic DNA sequence for channel catfish *myogenin*, *MYOD1* and *follistatin*. *Myostatin* gene-specific primers were developed from the published channel catfish *myostatin* sequence (Kocabas *et al.* 2002). These sequences were used to design primers to amplify full-length cDNA for each gene. Products amplified from cDNA of channel, blue and white catfish were cloned and sequenced as above.

Sequence comparisons

Homology searches were performed using BLASTN to confirm putative identity of each gene. Amino acid (AA) sequence was deduced from channel, blue and white catfish cDNA sequence. Exon–intron boundaries in channel catfish genes were verified by comparing genomic and cDNA sequences. Identity among channel catfish, blue catfish, white catfish, zebrafish and human AA sequences was calculated using CLUSTALW (Higgins *et al.* 1996; http://clustalw.genome.jp).

Expression analysis

Additional primers were designed to amplify a cDNA fragment (approximately 200–600 bp) to detect gene expression in cDNA prepared from channel catfish kidney, liver, muscle, heart, testes and spleen. Primer sets were designed to span an intron to eliminate potential confounding results from genomic DNA contamination (Table 1). A primer set was also designed to amplify channel catfish 18S ribosomal RNA (AF021880) as a positive control (reaction conditions listed in Table 1). Fragments produced from the tissue cDNAs were separated on a 2% agarose gel and visualized by ethidium bromide staining. Amplification of 18S and genes of interest were conducted in separate reactions because the expression of 18S was much higher than the expression of the genes of interest.

Linkage analysis

A short tandem repeat sequence was identified in each gene, and primers were designed to amplify a fragment-containing the repeat for each gene (Table 1). One primer of the set contained a 19 bp extension to permit addition of a fluorescent dye to the product (Table 1; Waldbieser *et al.* 2003). Fragments were amplified using a 3-step protocol (Table 1), separated on a ABI 3730XL and sized using GENEMAPPER software (Applied Biosystems). Genotypes were obtained for two reference families (Waldbieser *et al.* 2001) and outbred fish from commercial catfish operations. Linkage relationships were calculated using CRI-MAP 2.4 (Green *et al.* 1990).

Results and discussion

Sequencing

Complete genomic sequence for channel catfish *myogenin*, *MYOD1* and *follistatin* genes (GenBank accession numbers: AY534327–AY534329) were determined in the present research, whereas the channel catfish *myostatin* gene sequence was already available (Kocabas *et al.* 2002). Full-length cDNA sequences for all four genes were determined for blue and white catfish (AY562557, AY562555, AY574051, AY540992, AY540993, AY562556, AY574052 and AY540994).

The *myogenin* gene encoded a protein with 253 AA in channel, white and blue catfish compared with 256 in zebrafish and 225 in humans (AF202639 and AF050501). *Myogenin* gene structure (three exons, two introns, exon–intron boundaries) was identical between channel catfish, zebrafish and humans (Chen *et al.* 2000; Du *et al.* 2003). Amino acid identity with channel catfish was 99.6, 98.4, 82.2 and 48.8% for blue catfish, white catfish, zebrafish and humans, respectively (Fig. S1, Supplementary Material).

The *MYOD1* gene encoded a protein with 269 AA in channel, white and blue catfish compared with 275 in zebrafish and 320 in humans (AF318503 and AF027148). Gene structure of channel catfish *MYOD1* (three exons, two introns and exon–intron boundaries) was identical to that reported for other species (Chang *et al.* 1995; Tan & Du 2002). Two *MYOD1* genes have been reported in gilthead seabream (Tan & Du 2002) and rainbow trout (Rescan & Gauvry 1996; Delalande & Rescan 1999), but we did not identify a second catfish *MYOD1* gene. Amino acid identify with channel catfish *MYOD1* was 97.8, 98.5, 80.7 and 60.0% for blue catfish, white catfish, zebrafish and humans, respectively.

Channel catfish *myostatin* (Kocabas *et al.* 2002) encoded a protein of 389 AA, whereas blue and white catfish *myostatin* encoded proteins of 390 and 393 residues, respectively. These were longer than zebrafish (374) and human (375) *myostatins* (AY258034 and AF104922). A second *myostatin* gene has been identified in gilthead seabream, shi drum and the spotted green pufferfish (Maccatrozzo *et al.* 2001), but we have not identified a second *myostatin* gene in catfish. Amino acid identity with channel catfish was 96.9, 98.2, 80.0 and 60.8% for blue catfish, white catfish, zebrafish and humans, respectively.

Follistatin encoded a protein with 311 AA in channel catfish compared with 313 residues in white and blue cat-

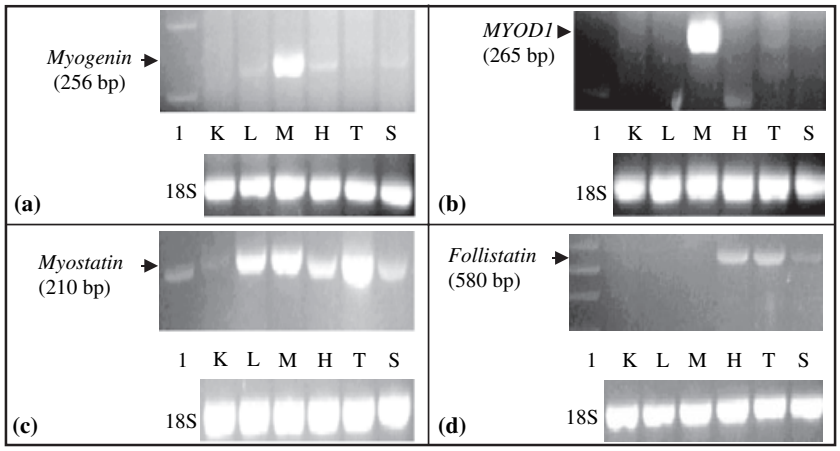

Figure 1 Tissue distribution of (a) *myogenin*, (b) *MYOD1*, (c) *myostatin* and (d) *follistatin* genes in cDNA from juvenile channel catfish. 1, 1 kb plus DNA ladder; K, kidney; L, liver; M, muscle; H, heart; T, testis; S, spleen.

fish, 322 in zebrafish (AF084948) and 317 in humans (NM006350). The genomic sequence indicated *follistatin* had five exons and four introns in channel catfish, but we found no evidence for alternative splicing of *follistatin* in channel catfish that has been reported for mammalian species (Shimasaki *et al.* 1988a,b; Michel *et al.* 1990; Nakatani *et al.* 2002). Amino acid identity with channel catfish was 98.7%, 97.7%, 72.3% and 67.2% for blue catfish, white catfish, zebrafish and humans, respectively.

Fish species with common reports of gene isoforms typically have had a genome duplication event during their evolutionary history, unlike catfish. Catfish may have isoforms for the genes we sequenced, but evidence for isoforms (significant sequence differences among individuals or size variation in amplification products) was not observed. However, more extensive research would be required to determine the presence of isoforms for these genes in catfish.

Tissue expression

Myogenin (Fig. 1a) and *MYOD1* (Fig. 1b) gene expression was limited to skeletal muscle in channel catfish as in other fish (Kobiyama *et al.* 1998; Rescan 2001; Tan & Du 2002; Du *et al.* 2003) and mammals (Sabourin & Rudnicki 2000). The *myostatin* gene was active in several tissues in channel catfish (Fig. 1c), in agreement with previous data for catfish (Kocabas *et al.* 2002) and other fish species (Ostbye *et al.* 2001; Rodgers & Weber 2001; Radaelli *et al.* 2003; Roberts & Goetz 2003). In contrast, *myostatin* is primarily expressed in skeletal muscle in

Table 2 Microsatellite polymorphism and linkage analysis of catfish myogenic regulatory genes.

Gene	Microsatellite	Location	Alleles	Number of fish genotyped	Heterozygosity	LG[1]	Closest marker	Recombinant fraction	LOD	Inform. meioses
Myogenin	$(TAA)_{19}$	Intron 2	238, 241, 244, 247, 250, 253, 268, 271, 274, 277, 288, 291	28	0.825	U23	*nRAMP*	0.11	14.4	100
MYOD1	$(CTTT)_{10}$	Intron 1	175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 215, 219	29	0.850	U14	*IpCG0063*	0.07	15.6	85
Myostatin	$(AT)_{37}$	Intron 1	269, 293, 299, 301, 303, 306, 308, 310, 313, 316, 319, 321, 323, 325, 328, 330, 343	22	0.928	U16	*IpCG0127*	0.20	4.7	60
Follistatin	$(ATT)_{15}$	5′-UTR	261, 264, 267, 270, 273, 276, 279, 282, 285, 288, 293	26	0.868	U15	*IpCG0156*	0	38.5	140

[1]LG, catfish linkage group (Waldbieser *et al.* 2001).

mammals (McPherron & Lee 1997), although minor levels of expression have been reported in other tissues (Ji *et al.* 1998; Sharma *et al.* 1999). The expression of *myostatin* in multiple tissues suggests its role may not be limited to control of muscle growth in fish. *Follistatin* gene activity was highest in heart and testis, lower in spleen, and not detected in kidney, liver and muscle (Fig. 1d). The lack of expression of *follistatin* in muscle could be related to developmental stage. The cDNA in the present research was prepared from a juvenile catfish, while data from other species suggests *follistatin*'s primary role in muscle development occurs during embryonic stages.

Linkage analysis

Genotype analyses revealed high levels of repeat polymorphism, 11–17 alleles per gene, in randomly selected catfish from commercial operations (Table 2). High levels of heterozygosity and large number of alleles in these fish were consistent with other catfish microsatellite loci (Waldbieser *et al.* 2001) and indicated the lack of sustained inbreeding in commercial catfish populations.

Allelic polymorphism in catfish reference populations permitted placement of the genes on the linkage map. Addition of *myostatin* to the map reduced a 29 cM gap on U16 to 21 cM and *follistatin* was in complete linkage to an existing anonymous marker (*IpCG0156*). However, *myogenin* and *MYOD1* were terminal markers and extended linkage groups U23 and U14 by 11.2 cM and 17.4 cM, respectively. This data suggests the current catfish linkage map (estimated at 2200 cM) could increase significantly as more markers are added.

A search of available vertebrate genomes (http://www.ncbi.nlm.nih.gov/mapview/) revealed synteny of *myogenin* and *nRAMP* in mouse but not in human or rat. Data were not available for zebrafish. Genetic distance between *myogenin* and *nRAMP* was approximately 33 cM in mouse compared with 11.2 cM in catfish. Addition of conserved genes to the catfish linkage map will permit identification of more syntenic regions with mammalian genomes to determine the utility of comparative mapping for catfish genetic analyses.

Supplementary material

The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/AGE/AGE1193/AGE1193sm.htm

Figure S1 Alignment of catfish peptide sequences for (a) *myogenin*, (b) *MYOD1*, (c) *myostatin* and (d) *follistatin*.

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